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Purification and characterization of cytochrome c_6 from *Acaryochloris marina*

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Abstract

Cytochrome c_6 , (cyt c_6) a soluble monoheme electron transport protein, was isolated and characterized from the chlorophyll d -containing cyanobacterium *Acaryochloris marina*, the type strain MBIC11017. The protein was purified using ammonium sulfate precipitation, ion exchange and gel filtration column chromatography, and fast performance liquid chromatography. Its molecular mass and pI have been determined to be 8.87 kDa and less than 4.2, respectively, by mass spectrometry and isoelectrofocusing (IEF). The protein has an alpha helical structure as indicated by CD (circular dichroism) spectroscopy and a reduction midpoint potential (E_m) of +327 mV versus the normal hydrogen electrode (NHE) as determined by redox potentiometry. Its potential role in electron transfer processes is discussed.

Keywords

Acaryochloris marina; Cyanobacteria; Cytochrome c_6 ; Photosynthesis; Chlorophyll d

Introduction

Acaryochloris marina, a unicellular marine cyanobacterium, is unique among oxygenic phototrophs in the fact that it uses chlorophyll d as its primary photosynthetic pigment. Compared to other chlorophyll species, chlorophyll d absorbs light at longer wavelengths (Miyashita et al. 1996; Hu et al. 1998; Schenderlein et al. 2008; Swingley et al. 2005; Tomo et al. 2008). In the environments in which *A. marina* grows, including associations with ascidians (sea squirts) and eukaryotic macro-algae and as a part of microbial mats in the Salton Sea, a California salt lake, other chlorophyll-containing species absorb most of the shorter wavelength light. This leaves unused near infrared light from 700 to 740 nm, which

A. marina can absorb efficiently with its unique chlorophyll *d* pigment (Miller et al. 2005; Bailleul et al. 2008; Cser et al. 2008; Ohasi et al. 2008; Renger and Schlodder 2008; Swingley et al. 2005; Swingley et al. 2008). The presence of this unusual pigment has made *A. marina* a species of interest for evolutionary analysis and possibly for bioenergy applications, and so we have begun investigating the proteins and complexes involved in its photosynthetic apparatus. One such protein is cytochrome *c*₆, (cyt *c*₆) which is involved in *A. marina*'s photosynthetic electron transport chain (Bailleul et al. 2008).

Cyt *c*₆, a small soluble monoheme protein, is responsible for transfer of electrons between the cytochrome *b*₆*f* complex to Photosystem I (PSI) in cyanobacterial and algal species (Bialek et al. 2008; Chida et al. 2007; Cho et al. 1999; De la Cerda et al. 1999; Inda et al. 1999; Proux-Delrouye et al. 2003; Steiner et al. 2000). The protein is categorized as a class I cytochrome with histidine and methionine serving as its fifth and sixth ligands (Diaz-Quintana et al. 2003; Marcaida et al. 2006; Medina et al. 1993; Worrall et al. 2007). It is a small protein, usually less than 100 amino acid residues in length and less than 10 kDa in molecular mass (Bialek et al. 2008; Campos et al. 1993; Steiner et al. 2000). The pI of the protein can vary widely among species; typically it is acidic in algae and unicellular cyanobacteria and basic in filamentous cyanobacteria (Inda et al. 1999; Medina et al. 1993). Among cytochromes, it has a high midpoint potential (*E*_m) ranging between 300 and 390 mV versus the normal hydrogen electrode (NHE; Bialek et al. 2008; Campos et al. 1993; Inda et al. 1999; Steiner et al. 2000). The difference in pI and *E*_m among species is thought to be due to changes in the charged residues that dock with cytochrome *f*, which hints at a possible co-evolution of cyt *c*₆ and cytochrome *f* (Diaz-Quintana et al. 2008; Kerfeld and Krogman 1998).

Different isoforms of cyt *c*₆ have been found during purification of various cyanobacterial species (Kerfeld and Krogman 1998). The genome of *A. marina* has been sequenced and shows two possible cyt *c*₆ isoforms annotated as *petJ* genes: AM1_2756 having 121 amino acids with a theoretical molecular weight of 10,759 Daltons for the gene product and a pI of 8.0 for the mature protein and AM1_3887 having 109 amino acids with a theoretical molecular weight of 10,819 Daltons for the gene product and a pI of 4.2 for the mature protein (Swingley et al. 2008).

In many cyanobacterial and algal species, plastocyanin (PC) a small blue copper protein, can functionally replace cyt *c*₆ in its electron transfer role (Bailleul et al. 2008; Chida et al. 2007; Cho et al. 1999; De la cerda 1999; Diaz-Quintana et al. 2003; Steiner et al. 2000). The sequenced *A. marina* genome also shows the presence of plastocyanin annotated as the *petE* gene: AM1_3369 (Swingley et al. 2008). When both proteins are present in a species, PC is typically the dominant protein expressed while cyt *c*₆ is progressively expressed under increasingly copper deficient conditions (Bialek et al. 2008; Campos et al. 1993; Cho et al. 1999; Diaz-Quintana et al. 2003; Inda et al. 1999; Worrall et al. 2007). In addition, PC and cyt *c*₆ are usually of similar size, pI, and *E*_m when both occur in the same species (Kerfeld and Krogman 1998; Medina et al. 1993). The predicted pI for the PC of *A. marina* is 4.34, which is very similar to the predicted value for the cyt *c*₆ gene product of AM1_3887. It has been suggested that the presence of these two unrelated proteins performing the same function in the same species has implications in photosynthetic evolution. When the Earth was still under anaerobic conditions, reduced iron was more soluble and readily available than reduced copper and cyt *c*₆ evolved first. However, when oxygen became plentiful, soluble oxidized copper was more readily available than oxidized iron and PC became the main electron transfer protein (Marcaida et al. 2006). This hypothesis is supported by the fact that only PC is present in this role in higher plants (Campos et al. 1993; Chida et al. 2007; De la Cerda et al. 1999; Diaz-Quintana et al. 2003, 2008; Inda et al. 1999; Kerfeld and Krogman 1998; Medina et al. 1993).

In addition, other modified cyt c_6 forms have recently been discovered in higher plants and the cyanobacterium *Synechococcus* sp. PCC 7002 (Bialek et al. 2008; Marcaida et al. 2006; Worrall et al. 2007). The form in higher plants has been designated cytochrome c_{6A} and differs from the typical cyt c_6 by the presence of a 12 residue loop and a lower redox potential. The lower potential precludes it from serving as an effective electron transfer agent between cytochrome b_6f and PSI and its function remains unclear (Marcaida et al. 2006; Worrall et al. 2007). *Synechococcus* sp. PCC 7002 has two genes *petJ1* and *petJ2*, which code for two different isoforms of cyt c_6 . The gene product of *petJ1* is a typical cyt c_6 , while that of *petJ2* has a substantially lower redox potential and its function is also unclear. It has been tentatively designated as a cytochrome c_{6c} form (Bialek et al. 2008).

Materials and methods

Growth conditions

The type strain MBIC 11017 of *A. marina* was grown in 16 L of culture in carboys for two weeks in Marine BG-11 media, a modified form of Blue-Green-11 media with 3.5% sea salt and enriched iron content equaling 0.016 g/L. Copper was present in the media as a trace metal at a concentration of 80 $\mu\text{g/L}$. The cultures were grown aerobically with air bubbling through a sterile filter at room temperature under a light intensity of 15–17 $\mu\text{mol/m}^2 \text{ s}$. Cells were harvested by centrifugation at 7,000 rpm. The resulting pellets weighed typically between 8 and 10 g.

Purification protocol

The harvested cell pellets were re-suspended in 50 ml of 0.75 M phosphate buffer pH = 7. The high salt concentration was required to stabilize phycobillisome proteins that were being isolated simultaneously with cyt c_6 . The proteinase inhibitor PMSF was added to 1 mM concentration to the buffer. In addition, DNase and magnesium chloride were added to 50 μM and 10 mM concentration, respectively, to the buffer. The cells were then broken using a bead beater (Biospec Products) with silicon carbide beads. The beads were added in a 1:2 ratio to the cell solution by volume. The bead beater was run in 20 s intervals and then placed on ice for 5 min to cool. The breaking and cooling steps were repeated for a total of six times.

Following the breakage step, the cells were centrifuged at low speed (3,850 rpm) at 15°C to remove beads and cellular debris. After low speed centrifugation, the resulting supernatant liquid was ultra-centrifuged at 44,000 rpm at 15°C for 2 h. The supernatant liquid from the ultra-centrifugation step was dialyzed overnight against 20 mM HEPES buffer pH = 7 at 4°C to exchange buffers before proceeding with further purification.

The dialyzed solution was then brought to 60% ammonium sulfate saturation by slowly adding ammonium sulfate while stirring. After all ammonium sulfate was dissolved, the solution was left to stir at 4°C for 30 min to ensure equilibrium had been reached. The solution was centrifuged at 10,000 rpm at 4°C for 15 min. The resulting supernatant liquid, which contained the cyt c_6 fraction, was then extensively dialyzed against 20 mM HEPES pH = 7 to remove the ammonium sulfate.

After dialysis, the solution was filtered successively with 0.45 and 0.22 μm filters before being loaded onto a QSPFF anion exchange column (GE Healthcare). After washing with binding buffer, the target protein was eluted with a step gradient of 0.1 M NaCl in 20 mM HEPES buffer pH = 7 at a flow rate of 2.5 ml/min. The fraction containing cyt c_6 appeared as a light pink band on the column. As the band moved near the end of the column, the eluent was monitored for cyt c_6 by measuring the UV-Vis spectra and looking for the

characteristic cytochrome peak at 415 nm. Collection of the eluent began once this characteristic peak appeared and ended when the UV–Vis spectra no longer showed any presence of this peak. The collected fraction totaled near 25 ml and was stored at 4°C.

The purification to this point was repeated over three harvests. The collected fractions from the 1st ion exchange chromatography (IEC) column were then pooled and concentrated with a 10-kDa cutoff Amicon filter to a final volume of 0.25 ml.

After concentration, the pooled fractions were loaded onto a Sephacryl-100 HR gel filtration column (GE Healthcare). The cyt c_6 fraction appeared as a pink band on the column as the sample was eluted at a flow rate of 0.5 ml/min with 20 mM HEPES buffer pH = 7. When the pink band neared the end of the column, the eluent was collected in 3 ml fractions. The fractions were monitored for cyt c_6 presence by measuring their UV–Vis spectra and monitoring the characteristic 415 nm peak. Peak fractions were collected and pooled together.

For the final purification step, the pooled fractions were loaded onto a high resolution 2nd IEC UNO Q1 column (Bio-Rad). The sample was eluted by a 1 ml/min flow rate of a linear gradient from 0–0.5 M NaCl in 20 mM HEPES buffer controlled using a Biologic DuoFlow System (Bio-Rad). The eluent was collected in 1 ml fractions and cyt c_6 presence was monitored by UV–Vis Spectroscopy.

UV–Vis spectroscopy

The UV–Vis spectra of the samples were taken during purification and of the final product with a Perkin Elmer Lambda 950 UV–Vis spectrophotometer and analyzed by Perkin Lambda UV WinLab Explorer software. The spectrum of the purified protein in its native reduced state was taken in 20 mM HEPES pH = 7. The spectrum of the oxidized species was taken by adding potassium ferricyanide to 1 mM to the purified protein sample.

SDS-PAGE

SDS-PAGE analysis was performed on the peak fraction collected from the 2nd IEC column to determine purity of the sample. The sample and Polypeptide Molecular Weight Standards (Biorad) were run on a 10–20% acrylamide gradient precast tris-tricine SDS-PAGE gel (Biorad) designed for peptides and small proteins at 30 V for 1 h and 100 V for 1 h using a Criterion Cell (Biorad) and an EC 150 power supply (EC Apparatus Corporation).

Isoelectrofocusing

The pI of cyt c_6 was determined by isoelectrofocusing (IEF). Purified cyt c_6 sample was run on an IEF gel (Bio-Rad) with a pH gradient 3–10 with IEF standards (Bio-Rad). The gel was run at 100 V for 1 h with IEF cathode and anode buffer (Bio-Rad). The theoretical pI of the protein was calculated from the amino acid sequence using the compute pI/MW tool (http://a.expasy.org/tools/pi_tool.html).

Mass spectrometry

MALDI-TOF mass spectrometry was performed on the undigested purified cyt c_6 using a 4700 Proteomics Analyzer and Science Explorer Software (Applied Bio-systems). For the undigested protein, a sample was taken directly from the purified cyt c_6 and analyzed with the instrument to determine the mass of the intact protein. In addition, cytochrome c (cyt c) from equine heart (Sigma) was used as an internal standard to calibrate the instrument to determine the experimental error for the molecular mass.

LC-MS/MS was used to confirm the identity of the purified protein as cyt *c*₆ from *A. marina* using a reverse phase C₁₈ column custom packed with silica media (Microohm Bioresources) and a LTQ-orbitrap mass spectrometer (ThermoScientific). The purified protein was tryptic digested following an in-solution protocol. The protein was first denatured by boiling, after which trypsin protease digestion solution (Sigma) was added to the sample. The sample was then digested overnight at 37°C. After digestion, the sample was analyzed by the mass spectrometer following a protocol previously established in our lab (Wen et al. 2009).

The theoretical mass of the intact protein and tryptic peptides was calculated using compute pI/MW tool and FindPept tool (http://ca.expasy.org/tools/pi_tool.html; <http://ca.expasy.org/tools/findpept.html>). Signal peptide prediction was performed using SignalP 3.0 Server to aid in the comparison of the experimental and theoretical masses (<http://www.cbs.dtu.dk/services/SignalP/>). The MASCOT program was used to search the NCBI database for matches with the product ions from the MS/MS analysis (<http://www.matrixscience.com/>).

Circular dichroism spectroscopy

The circular dichroism (CD) spectrum of cyt *c*₆ at a concentration of 0.1 mg/ml in 10 mM phosphate buffer pH = 7 was determined with a JASCO J-815 CD spectrometer. The raw data were converted to molar ellipticity using Spectra Manager II software. Secondary structure analysis was performed using CD PRO Software and its analysis programs SELCON3, CDSSTR, and CONTIN (<http://lamar.colostate.edu/~sreeram/CDPro/main.html>).

Redox titration

A redox titration was performed to determine the E_m of cyt *c*₆. Potential was controlled using a CH 620 C potentiostat (CH Instruments) and spectral changes of cyt *c*₆ upon reduction and oxidation were monitored at 553 nm in a Perkin Elmer Lambda 950 UV-Vis spectrophotometer. The electrodes used were a platinum gauze working electrode, Ag/AgCl reference electrode, and platinum wire auxiliary electrode. 0.1 M NaCl was added as the supporting electrolyte to the cyt *c*₆ sample in 20 mM HEPES buffer pH = 7. Potassium ferricyanide, dimethyl phenylene diamine, Fe(III) EDTA, and phenazine ethosulfate were all added at 20 μ M to serve as redox mediators.

The fraction of cyt *c*₆ reduced at each potential was determined by the changes in absorbance according to Eq. 1 where χ is the fraction reduced at the respective potential, A is the absorbance at 553 nm at the respective potential, A_o is the absorbance of the fully oxidized species at 553 nm, and A_r is the absorbance of the fully reduced species at 553 nm.

$$\chi = (A - A_o) / (A_r - A_o) \quad (1)$$

The fraction reduced was then plotted against potential (vs. NHE) in ORIGIN and fitted to a modified Nernst equation given in Eq. 2 where χ is the fraction reduced at a given potential, E is the given potential, and E_m is the midpoint potential. These equations were derived using the method developed by Dutton (1978). A best fit for the midpoint potential parameter was obtained using ORIGIN's iterative nonlinear regression analysis.

$$\chi = 10^{(E - E_m)/59} / (10^{(E - E_m)/59} + 1) \quad (2)$$

Results

UV-Vis spectroscopy

UV-Vis spectra of the purified sample in both its native reduced state and oxidized state are shown in Fig. 1. The reduced species showed the characteristic Soret band at 415 nm, alpha band at 553 nm, beta band at 520 nm, and delta band at 316 nm along with a band at 275 nm representing total protein. The ratio of the absorbance at the 275 nm band versus at the 415 nm band is 0.23. Upon oxidation, the alpha and beta bands disappear and are replaced with a broader band with a peak at 527 nm. The Soret band decreases in intensity and blue-shifts to 409 nm. Finally, the band at 316 disappears and a new band appears at 357 nm.

SDS-PAGE

The SDS-PAGE of the peak fraction from the 2nd IEC column showed only one clearly distinct band between the 14 and 6.5 kDa markers indicating a highly pure protein (Fig. 2).

Isoelectric focusing

The pI of cyt *c*₆ was determined to be less than 4.2 based on the IEF gel run with standards (Fig. 3). The predicted pI's for the two different cyt *c*₆ isoforms encoded by the AM1_2756 and AM1_3887 genes are 8.0 and 4.2, respectively indicating the gene product of AM1_3887 is the isoform expressed under our growth conditions.

Mass spectrometry

The MALDI-TOF spectrum of the whole protein showed peaks at 12360, 8868, 6180, and 4435 Da (Fig. 4). The set of peaks at 8,868 and 4,435 Da represent the masses of the singly charged cyt *c*₆ plus the mass of a proton and of the double charged cyt *c*₆ plus the mass of two protons, respectively, while the set of peaks at 12,360 Da and 6180 Da represent the masses of the singly charged cyt *c* plus the mass of a proton and of the double charged cyt *c* plus the mass of two protons, respectively. The known mass for the internal standard cytochrome *c* from equine heart is 12,362 Da giving an experimental error in the mass accuracy of ± 4 Da. Therefore, the experimentally determined mass of cyt *c*₆ is $8,867 \pm 4$ Da.

The signal peptide analysis predicted a signal sequence cleaved between Ala 25 and Ala 26 residues. After subtracting the mass of this sequence and adding the mass of a *c* type heme (616 Da), the theoretical mass of the protein is predicted to be 8,867 Daltons. Thus the experimental and theoretical mass of the protein are in agreement.

The predicted peptide fragments of the AM1_3887 isoform from tryptic digestion are 2276, 2673, and 2807 Da, while those from the AM1_2756 isoform are 5321, 4255, 763, 629, and 503 Da. A peak at 2276 Da representing the tryptic digested peptide of ADALSANGMDSADAIINQVTNGK from the AM1_3887 isoform was found with the Orbi-trap mass spectrometer which was then further analyzed by MS/MS using the ion trap part of the instrument. The product ions from the MS/MS analysis (Fig. 5) were then used in MASCOT to search for matches from the NCBI database. The first hit retrieved by MASCOT matched the *A. marina* cyt *c*₆ protein encoded by the AM1_3887 gene with a probability based mowse score of 2,096. The other hits retrieved by MASCOT were matches from other bacteria all with less significant mowse scores of lower than 100. This confirms that the purified protein is cyt *c*₆ from *A. marina* and indicates that the isoform isolated during our purification is the one coded by the AM1_3887 gene, which is also in agreement with the IEF data.

CD spectroscopy

The CD spectrum of cyt c_6 was measured at 0.1 mg/ml concentration in 10 mM phosphate buffer pH = 7 (Fig. 6). Two peaks appear near 209 and 222 nm which is characteristic of an alpha helical structure. Secondary structure analysis predicted the overall structure to be alpha helical. The average structure given by the CD PRO analysis was a 66.1% alpha helical structure with most of the remaining structure constituted from undetermined or random folds at 25.6%. The analysis indicated a small amount of beta sheets (3%) and turns (4%) present in the sample.

Redox titration

The E_m for cyt c_6 as determined by a redox titration was +327 mV versus NHE. The standard error in the midpoint potential is ± 2 mV obtained by the ORIGIN fitting analysis. The redox titration plot shows a reversible Nernstian behavior corresponding to a one electron oxidation/reduction reaction (Fig. 7).

Discussion

Cyt c_6 from *A. marina* has been purified using ammonium sulfate fractionation, column chromatography including ion exchange and gel filtration, and FPLC. Only one cyt c_6 isoform, corresponding to the AM1_3887 gene, was detected during the purification. In addition, no plastocyanin was detected during the purification. This result is in contrast with the recent analysis performed by Bailleul et al. (2008) in which they concluded that plastocyanin, not cyt c_6 , was the majority soluble electron carrier between cytochrome b_6f and PSI in *A. marina*. In their study, they found that after a series of actinic flashes that absorbance of *A. marina* whole membrane samples were photo-bleached at 554 nm, where cyt c species absorb strongly. They concluded that this bleaching could be due to the oxidation of the cytochrome b_6f complex alone or oxidation of both the complex and cyt c_6 . Since, 75% of the bleaching occurred after the first flash corresponding to one charge separation, it was concluded that the pool of electron donors to PSI associated with the bleaching was not in excess with PS I. Therefore, cyt c_6 was considered unlikely to be the soluble electron carrier because the carrier is typically in an excess of 3:1 to PSI. However, this evidence against the presence of cyt c_6 is indirect and no direct evidence for plastocyanin was presented in their analysis since its absorption spectrum is weak and broad and overlaps that of PSI (Bailleul et al. 2008).

The detection of only cyt c_6 during our purification protocol suggests that in fact the cytochrome species is a major soluble electron carrier. This conclusion would suggest along with the findings of Bailleul et al. (2008), an unusual case of an electron carrier being present in a substoichiometric amount relative to PSI. However, *A. marina* was grown in media with a higher concentration of iron in our study than that of the Bailleul et al. (2008) study. Quantitative measurements of the levels of plastocyanin and cyt c_6 under different growth conditions would help clarify this situation.

While *A. marina* is unique among cyanobacteria in using chlorophyll d as its primary pigment, its cyt c_6 appears to be more typical of other unicellular cyanobacteria. It is a small acidic protein with molecular mass of 8.85 kDa and a pI near 4. As indicated by CD Spectroscopy, it has an overall alpha helical structure with an estimated 66.1% percentage of its secondary structure being alpha helical. This percentage is slightly higher compared to those of cyt c_6 from other organisms with known crystal structures. Cyt c_6 from *Acaryochloris maxima*, *Poryphora yezoensis*, *Phaeodactylum tricornutum*, *Cladophora glomera*, and *Scenedesmus obliquus* have alpha helical secondary structure percentages of 59, 61, 57, 59, and 57%, respectively (<http://www.pdb.org/pdb/home/home.do>). In addition,

it has a high midpoint potential as indicated by redox titrations with an E_m equal to $+327 \pm 2$ mV, which is similar to the value found in other cyanobacteria. This similarity has some bearing on the recent debate of the value of the E_m of PSI from *A. marina*.

Hu et al. (1998) reported the E_m for P740 in PSI in *A. marina* to be +335 mV, substantially lower (~100 mV) than PSI of other cyanobacteria. The value was determined by monitoring flash-induced absorbance changes at 740 nm of purified PSI complexes held at different potentials by means of chemical titration. Since the PSI of *A. marina* absorbs light at a 0.1 eV lower quantum energy than the PSI of chlorophyll *a* containing cyanobacteria and plants (absorption at 740 nm compared to 700 nm), there may be a decrease in the thermodynamic driving force for the primary electron transfer steps in *A. marina* compared to chlorophyll *a* containing species (Hu et al. 1998). The finding of Hu's study (1998) suggested that the decrease in E_m for the PSI of *A. marina* may compensate for this apparent decrease in the driving force.

However, recent studies by other research groups have determined the E_m of P740 in PSI of *A. marina* to be much higher ranging from +425 to +450 mV, similar to that found in other cyanobacteria (Schelenderin et al. 2008; Bailleul et al. 2008; Tomo et al. 2008). Schelenderin et al. 2008 found the E_m to be +450 mV by following flash-induced absorbance changes at 824 nm at varying potential by chemical titration. Bailleul et al. 2008 followed absorbance changes at 740 nm by both chemical and electrochemical titration and reported a value of +425 mV. Finally, Tomo et al. (2008) determined a value of +439 mV by following absorbance changes at different wavelengths including 740 nm by electrochemical titration. Since the primary electron acceptor is not known for *A. marina*, it has been suggested that it could be a chlorophyll *a* instead of *d* and that changes in the E_m of the primary acceptor species could compensate for the difference in the energy of light absorbed (Bailleul et al. 2008; Tomo et al. 2008). Furthermore, it could be more important to maintain the high E_m for PSI to make it thermodynamically favorable for electron transfer between it and its soluble electron donor, cyt *c*₆ or plastocyanin (Bailleul et al. 2008; Tomo et al. 2008). While it could be possible there is a shift in the E_m of the donor as well, this is not the case found in our study where the E_m of cyt *c*₆ was determined to be +327 mV, within range of the E_m of other cyanobacteria cyt *c*₆ (Bialek et al. 2008; Campos et al. 1993; Diaz-Quintana et al. 2008; Inda et al. 1999; Kerfeld and Krogman 1998; Steiner et al. 2000). This value would be too positive for a favorable transfer between it and PSI if the Hu et al. value is correct, but would have a similar difference in potential to PSI of other cyanobacteria, +324 mV (cyt *c*₆) and +423 mV (PSI) for *Thermosynechococcus elongates* and +320 mV (cyt *c*₆) and +450 mV (PSI) for *Synechocystis* sp. PCC 6803, if the +425 to 450 mV range for *A. marina* PSI is correct (Cho et al. 1999; Hou and Mauzerall 2006; Nakamura et al. 2005; Proux-Delrouyre et al. 2003).

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Abbreviations

cyt <i>c</i> ₆	Cytochrome <i>c</i> ₆
FPLC	Fast protein liquid chromatography
IEF	Isoelectrofocusing

CD	Circular dichroism
E_m	Midpoint potential
NHE	Normal hydrogen electrode
<i>A. marina</i>	<i>Acaryochloris marina</i>
PSI	Photosystem I
PC	Plastocyanin
IEC	Ion exchange chromatography
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
LC-MS/MS	Liquid chromatography tandem mass spectrometry
χ	Fraction reduced
A	Absorbance
A_o	Absorbance of fully oxidized species
A_r	Absorbance of fully reduced species
E	potential

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Fig. 1.
UV-Vis Spectra of purified cyt *c*₆ from *A. marina* with the solid line representing the reduced form and the dashed line representing the oxidized form

Fig. 2. 10–20% Gradient SDS-PAGE Gel of cyt c_6 from *A. marina*. *Lane 1* cyt c_6 sample from 2nd IEC; *Lane 2* Polypeptide Molecular Weight Standards

Fig. 3.
IEF Gel of purified cyt *c*₆ from *A. marina*. Lane 1 IEF Standards; Lanes 2 and 3 purified cyt *c*₆

Fig. 4. MALDI-TOF Spectrum of isolated whole mass cyt c_6 from *A. marina* with cyt c from equine heart as an internal standard

Fig. 5.
 LC-MS/MS Product ions from (a) Tryptic digested peptide
 ADALSANGMDSADAIINQVTNGK and (b) and their ms/ms Mass spectra from purified
 cyt *c*₆ from *A. marina*

Fig. 6.
CD spectrum of purified cyt *c*₆ from *A. marina*

Fig. 7. Redox titration of purified cyt c_6 from *A. marina* oxidative titration (black squares), reductive titration (white triangles), and Nernst fit (straight line)